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Review

Biophysics of sphingolipids II. Glycosphingolipids: An assortment of multiple structural information transducers at the membrane surface

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Abstract

Glycosphingolipids are ubiquitous components of animal cell membranes. They are constituted by the basic structure of ceramide with its hydroxyl group linked to single carbohydrates or oligosaccharide chains of different complexity. The combination of the properties of their hydrocarbon moiety with those derived from the variety and complexity of their hydrophilic polar head groups confers to these lipids an extraordinary capacity for molecular-to-supramolecular transduction across the lateral/transverse planes in biomembranes and beyond. In our opinion, most of the advances made over the last decade on the biophysical behavior of glycosphingolipids can be organized into three related aspects of increasing structural complexity: (1) intrinsic codes: local molecular interactions of glycosphingolipids translated into structural self-organization. (2) Surface topography: projection of molecular shape and miscibility of glycosphingolipids into formation of coexisting membrane domains. (3) Beyond the membrane interface: glycosphingolipid as modulators of structural topology, bilayer recombination and surface biocatalysis.

Keywords: Ganglioside; Glycosphingolipid-enriched domain; Glycosphingolipid in membrane topology; Membrane fusion; Glycosphingolipid–phospholipid interaction; Epifluorescence microscopy; Brewster angle microscopy; Phospholipase; Sphingomyelinase

Contents

Abbreviations: GSLs, glycosphingolipids; Cer, N-acysphingosine (ceramide); Brain neutral GSLs and gangliosides (ganglio-series) have the same hydrocarbon moiety composition, sphingosine-linked fatty acids are over 85% stearic, arachidic and nervonica acid, sphingosine base is over 82% 18:1 and 20:1 (4-sphinegenine); GalCer, Galβ1–1′Cer; GlcCer, Glcβ1–1′Cer; LacCer, Galβ1–4Glcβ1–1′Cer; Gg3Cer, GalNAcβ1–4Galβ1–4Glcβ1–1′Cer; Gg4Cer (asialo-GM1), Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1′Cer; GM3 (II^3NeuGc-LacCer), NeuGcα2–3Galβ1–4Glcβ1–1′Cer; GD3 (II^3 $(NeuAc)_2$-LacCer), NeuAcα2–8NeuAcα2–3Galβ1–4Glcβ1–1′Cer; GM2 (II^3 NeuAc-$GgOse_3$Cer), GalNAcβ1–4Gal(3–2αNeuAc)β1–4Glcβ1–1′Cer; GM1 (II^3NeuAc-$CgOse_4$Cer), Galβ1–3GalNAcβ1–4Gal(3–2αNeuAc)β1–4Glcβ1–1′Cer; GD1a (IV^3NeuAc, II^3NeuAc-$CgOse_4$Cer), NeuAcα2–3Galβ1–3GalNAcβ1–4Gal(3–2αNeuAc)β1–4Glcβ1–1′Cer; GT1b (IV^3NeuAc, II^3 $(NeuAc)_2$-$CgOse_4$Cer), NeuAcα2–3Galβ1–3GalNAcβ1–4Gal(3–2αNeuAc8–2αNeuAc)β1–4Glcβ1–1′Cer; Sulf, HSO_4-3Galβ1–1′Cer; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; CHOL, cholesterol; SM, sphingomyelin; HI, Hexagonal I phase; HII, Hexagonal II phase; PLA_2, Phospholipase A_2; PLC, Phospholipase C; SMase, Sphingomyelinase; MBP, Myelin Basic Protein; T_m, melting temperature; IR, Infrared Spectroscopy; EPR, Electron Paramagnetic Resonance; AFM, Atomic Force Microscopy; BAM, Brewster Angle Microscopy

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1. Introduction

More than a century has gone by from the time Johan L.W. Thudichum, a student of von Leibig, discovered "cerebroside" in 1884, a lipid with uncommon properties found in solvent extracts of human brain. He also reported that cerebroside contained a unique insoluble long-chain base that could be liberated by barium hydroxide hydrolysis of the lipid. In a historical account given in the introductory note of a symposium on the biological effects of sphingolipids in the nervous system [1], a statement by Thudichum is reported defining the substance as of "an alkaloid nature, and to which, in commemoration of the many enigmas which it presented to the inquirer I have given the name of Sphingosin". For the name, he was inspired from the enigmatic Sphinx for which, according to legend, failure to solve its riddle incurred the penalty of death. Actually, it could be said that Thudichum might have been somehow prophetic regarding some of the earliest neurochemists because, although much information has been collected over the years, the riddle regarding the structural molecular behavior and dynamics in relation to biomembrane function of the fascinating family of sphingolipids is not completely solved and still eludes understanding in many aspects.

The full stereochemical structure of sphingosine and cerebroside, subsequently paving the way to that of other sphingolipids, had to wait more than 60 years to be solved since its discovery [2,3]. Most of the early studies on sphingolipids followed Thudichum's lead in using nervous tissue as a source, and improved methods for their purification and characterization revealed an extraordinary variety of compounds. Many were denominated with ingenious names that, for the sake of mnemonics, combined chemical and structural properties (i.e., ceramides (Cer), sulfatides (Sulf)) with their neural origin (i.e., sphingomyelin (SM), cerebrosides). A major impetus to study glycosphingolipid chemistry and biochemistry was the discovery of an abnormal lipid called "substance X" in brain tissue from a child with infantile amaurotic idiocy (Tay–Sachs disease) as the first known member of the ganglioside family discovered in 1935 [4]; the structure of Tay–Sachs ganglioside (GM2) was firmly established only by 1963 [5]. After the first evidences of the possible involvement of gangliosides in brain function [6], great efforts were dedicated to understand their function in nerve membranes, a quest that still remains largely unsolved. After another quarter century, the chemical structures and metabolic pathways of several glycosphingolipids (GSLs) began to be elucidated and systematized [7,8], a process that continues for many new components.

These lipids are constituted by a hydrocarbon portion consisting of a 18–20 carbons sphingosine base with an amide-linked fatty acyl chain that can generally be longer [9], but also shorter than the sphingosine moiety (ranging from 2 to 26 carbons in length [10]) to form the basic structure of Cer. Attachment by glycosidic linkage to the hydroxyl group in carbon 1′ of Cer of single carbohydrates or oligosaccharide chains of different complexity leads to the GSLs. These lipids are ubiquitous components of animal cell membranes. In most cells, there are simple neutral GSLs with one or two sugar residues such as glucose or galactose, intermediate molecular weight GSLs containing N-acetylglucosamine or N-acetylgalactosamine as well as glucose and galactose, some of which may have terminal or branched fucose or N-acetylneuraminic acid residues, and high molecular weight GSLs that may contain 20 or more glycose residues; those with more than six sugar residues generally have a repeating unit that forms the skeleton of the oligosaccharide chain and to which other sugars and N-acetyl or N-glycolyl-neuraminic acid can be attached. The pattern of GSLs in the nervous system is developmentally regulated, the various sphingolipid precursor–product relationships and specific enzymatic steps for GSLs and ganglioside biosynthesis have been well characterized [7,8,11]. The genetically regulated expression of the biosynthetic enzymes and their direct interaction with other regulatory proteins, along the membrane recycling pathway [12], have a fundamental participation in the metabolic regulation of the synthesis of these lipids [7,8]. Nevertheless, the fundamental factors at the local molecular level that underlay the separation of closely related biochemical pathways remain obscure while some hints in this regard, based

on their basic biophysical properties, may begin to emerge [13].

Several GSLs, or their phosphorylated or sulphated derivatives, have been implicated in various cell functions [1,14–17]. The major proportion of GSLs is located at the outer (extracellular) half of the bilayer where they are accessible for interactions with lectins, toxins, hormones, viruses and other external ligands (see [1] and references therein). However, they are not restricted to the outer membrane surface and a certain proportion is found in the inner half of bilayer lipid vesicles [18,19], neuronal membranes [20], bound in the cytosol to soluble cytoplasmic proteins [21,22], enzymes [20], associated to cytoskeletal elements [23], and in nuclear membranes [24]. Some GSLs are major components of specialized membranes of the nervous system and its content can be dramatically altered in neurological diseases, tumorigenesis and neurotopathological processes [1,25–28]. Gangliosides are specially enriched in neuronal membranes where they can represent between 2 and 10% of total lipids and where more than 30% of the total sialic acid is contributed by the ganglioside pool [27].

The diversity obtained by random combination of possible structures indicates that there could be millions of different compounds containing a core of 5 sugar residues [29]. By additionally considering independent variations in the hydrocarbon moiety, an enormous number of structural possibilities (with surely different biophysical properties) come about to hopelessly confront our intellect. Fortunately, the biosynthetic enzymes are specific, act under strict structure- and membrane-dependent control, and do not add sugar residues at random so that the number of actual possibilities is greatly reduced. However, an impressive variety of oligosaccharide chains occur in the more than 300 GSLs species characterized to date, with the number still growing. The challenge to unravel the molecular and functional significance for membrane function of this huge structural diversity has attracted the attention of biochemists, cell biologists and neuroscientists. However, for many years, most biophysicists dedicated little attention to sphingolipids in general and to GSLs in particular, especially for the more complex components, compared to the amount of studies done with glycerophospholipids. This was unfortunate since elucidation of the cellular function of GSLs can eventually be achieved only when their biochemical and structural properties are integrated within the dynamic membrane structure and, for this, biophysical approaches are unavoidable. The relative paucity of biophysical studies might be ascribed to at least some of the following causes such as the complexities involved in obtaining structurally defined synthetic derivatives and difficulties for isolation of many natural compounds in relatively large amounts. Another likely reason is the need for extremely careful purification and conservation conditions in order to obtain reproducible results (even by a same laboratory) with sensitive biophysical techniques. In fact, very small amounts of impurities that usually escape detection by routine checking (but that alter conspicuously their biomembrane behavior) are frequently present in reputedly pure preparations and moreover in commercial sources [30]. Besides, spontaneous physico-chemical time-, temperature-, hydration-, and concentration-dependent metastable polymorphism and structural transformation markedly change the ganglioside properties, even for well-purified preparations [30–34]. This is due to the extraordinary amplification capacity of most GSLs to alter supramolecular topology and structural stability of biomembranes in very small proportions (i.e., below a few mol%) [35–39].

Reviews were published from about the 1990s that summarized relevant findings on individual properties of specific GSLs in model membranes, in cellular function, and on their role as signaling modulators in the nervous system [1,12,14,15,40,41]. However, integration of their diverse and synergic biophysical properties within the molecular, supramolecular and topological membrane dynamics has been scarce. Even today, most studies on the biophysical properties of "GSLs" and "gangliosides" are based mainly on results obtained with monohexosyl- or dihexosyl-Cers or with ganglioside GM1 (or even speculated from the properties of Cer!). However, results should not be lightly extrapolated to all GSLs in general because the self-aggregation, molecular packing, phase state, and interactions of different GSLs with other membrane lipids and proteins is markedly different (see below). The neglect of this fact for quite some years has been unfortunate, confusing, and introduced rather gross misconceptions, specially in the cell biology field where biophysical properties and concepts are difficult to be timely incorporated. Perhaps, the powerful and intuitive Aristotelian reductionist conception that brought in successful sequential analysis of mechanisms dissected to their tiniest pieces has gradually impaired our capacity for an integrated interpretation of concomitant phenomena occurring over different scales of complexity [42]. The first paper regarding GSLs that attempted a hint in this direction was published 25 years ago [43], further advances were subsequently reported [44] and findings during the last decade were recently covered [13]. From the integrated view innovative concepts emerge on their effects in membrane structural dynamics. Different laboratories reported additional data confirming, recognizing explicitly or implicitly, and in occasions "rediscovering" many early results with different systems. However, consideration of the physico-chemical properties of GSLs as integrated codifiers, amplifiers and modulators of lateral and transverse molecular information exchange at the membrane level continues to be scarce.

In our opinion, most of the advances over the last decade on the biophysical behavior of GSLs can be organized into three related aspects of increasing structural complexity; we will integrate them within the general framework of molecular-supramolecular information transduction in biomembranes [13]: (1) intrinsic codes: local molecular interactions of glycosphingolipids translated into structural self-organization. (2) Surface topography: projection of molecular shape and miscibility of glycosphingolipids into formation of coexisting membrane domains. (3) Beyond the membrane interface: glycosphingolipid as modulators of structural topology, bilayer recombination and surface biocatalysis.

1.1. Molecular-to-supramolecular information transduction in the membrane scenario

GSLs do not escape the general biophysical constraints underlying the structural dynamics of membranes and we consider essential to first place their molecular properties in a proper stage. It has always been difficult to represent the complex molecular events involving many simultaneous interactions and local biocatalytic reactions that modify membrane composition, miscibility–immiscibility processes, thermodynamic and mechanical dissipation of tensions, and the structural outcome underlying biomembrane information transduction. The easy solution has been to oversimplify by representing molecular properties and influences in isolation and independently. The problem is not oversimplification itself but that its overuse brings about the detrimental result of ending up substituting reality for fiction, with erroneous concepts that may remain entrenched in their use for many years. As well known by most serious investigators, events in membranes do not follow the simple illustrations usually represented in biochemistry and cell biology university textbooks but involve dynamic transmission of information over different levels of complexity.

Though not always recognized, practically all we know on the structural dynamics of cell membranes was derived from a rich background of a few biophysical properties of membrane molecules obtained with model systems. This has shown that molecular information transduction involves much more than biochemically intertwined sequential reactions, receptor–ligand recognition, phosphorylation cascades and activation-inhibition of membrane-associated enzymes. The whole membrane structure, and beyond, is involved since each of these effects occur with changes of chemical structure, molecular configuration, or associations. It is thus thermodynamically unavoidable that they become amplified, balanced, translated and modulated through variations of molecular interactions (thus determining lateral miscibility, phase state and surface topography) and molecular geometry, surface electrostatics and viscoelastic tensions (thus controlling interfacial curvature and long-range communication across the lateral and transverse planes of the membrane), that take place over different dimension scales.

The concept of scale dimension is rarely considered but it is central to understanding the molecular behavior of biomembranes. On a molecular scale, the very slow transfer of membrane molecules to the aqueous environment, compared to the rate of lateral diffusion, means that the anisotropically restricted membrane surface behaves nearly as a closed system with a fixed bulk composition for relatively long (in terms of molecular events) periods of time (minutes to hours). Within this context, most of the "average" surface parameters are measured over a space-time scale that involves a sufficient number of molecules such that the individual molecular fluctuations are small. For biomembranes, the structure can only be considered to follow some "continuum" law in the two-dimensional plane since the third dimension, thickness, represents a structural and thermodynamic discontinuity. The fluctuations of a surface property are inversely proportional to the square root of the number of molecules considered within the scale of the continuum. As previously noted [45], for a molecule with an average cross-sectional area of about 1 nm^2 , and exhibiting fluctuation of this value of less than 1% , the valid surface size over which the measured mean molecular area is meaningful requires to be 0.01 μm^2 or larger. Consequently, average surface properties regarding the intermolecular organization represent the ensemble of many fast and local molecular events, integrated over a scale that spans at least fractions of μm, relatively long times, and covers the composite thickness dimension of that membrane region. Thus, interpreting the molecular codes underlying the membrane structural dynamics requires insights into the elusive structural–temporal dimension accurately denominated the "mesoscopic level" [46]. In this restricted zone, events take place beyond the size and fluctuations of individual moieties, while not yet in the macroscopic environment and sizes of functional membrane organelles.

One of the major parameters that influences, and responds, to variations of the molecular packing is the two-dimensional (lateral) surface pressure acting on the molecules at the interface. It arises mainly from the balance between hydrophobic effects that entropically tend to separate out non-polar moieties from the aqueous phase (thus forcing the energetically unfavorable formation of a hydrocarbon–water interface) while simultaneously overcoming the repulsive tendency to pack of the polar head groups [47]. The surface pressure represents a time- and space-averaged parameter for which the amplitude and frequency of fluctuations depend on the molecular thermal energy, in-plane elasticity and surface viscosity [48,49]. Besides homo- or hetero-tropic interactions, the average molecular area exhibited by a defined class of GSLs varies according to the surface hydration, state of protonation, ion binding, and the lateral surface pressure [43,50].Thus, it is quite inevitable that the molecular area and dipole potential density also fluctuate within a defined range about the average value, in correspondence with the surface pressure, either in-phase or out-of-phase depending on the surface elasticity and/or retardation by viscoelastic relaxation [43,44,47,51,52]. The values of "average" monolayer surface pressure that have been correlated to bilayers and natural membranes are in the range of 30–35 mN/m [44,47,49,53,54]. However, the query on what could be the surface pressure representing that corresponding to a natural membrane has, strictly speaking, little meaning because the mean value actually exhibits large fluctuations that can span more than 15 mN/m [48]. Those fluctuations occur in the time range from micro- to milli-seconds depending on surface compressibility and on the average size of the domain over which the fluctuation is felt [55]. The actual value of the surface pressure, and its fluctuations in time, is due to the transfer of momentum during collisions between the molecules forming the surface and by surface osmotic effects due to the lower water chemical potential at the interface. Thus, the lateral pressure is influenced by the average (and thus also fluctuating) thermal energy, steric hindrance, hydration, electrostatic, repulsive or cohesive interactions in the membrane [48,49]. Furthermore, variations of the mean molecular area directly translate to

changes of interfacial polarization density thus controlling surface electrostatics.

Topographically, the time-dependent lateral pressure waves are reflected in the average phase state and its transition cooperativity; the in-plane elasticity and vectorial shear tensions can adsorb, dampen or amplify those variations. If the compressibility of the surface phase is approximately isotropic along the lateral plane (i.e., in the absence of shear viscosity), the stress generated by pressure fluctuations may be propagated as more or less uniformly dampened oscillations. However, if less deformable barriers (such as the presence of segregated domains with more condensed phase states) are encountered, changes of interactions and/or isothermal phase transitions could be expected along the perturbation path. The kinetics for formation of phase segregated domains having a relatively small number of molecules, or large scale (critical) phase fluctuations, is well within the time range of surface pressure fluctuations [55]; this is about 2–4 orders of magnitude faster than the catalytic rate of membrane-associated enzymes [56]. On the other hand, if the lipid polar head groups contain net charges, pressure induced fluctuations of the molecular packing will also be transduced to concerted fluctuations of the surface electrostatics, both laterally and perpendicularly to the membrane plane, coupled to oscillating variations of the double layer potential extending into the aqueous medium depending on the electrolyte concentration [49,57,58]. The proven existence of this type of synergic, and oligosaccharide chain-dependent, effects is the very reason of why the properties and interactions of their individual moieties (i.e., the Cer portion, specific carbohydrates) cannot be assumed to remain similar in the different GSLs (see below).

1.2. The intrinsic codes: local molecular interactions of GSLs translated into structural self-organization

The varied chemical features of GSLs confer unique properties for information exchange in membrane dynamics brought about by metabolically-driven, relatively simple and specific, selective chemical changes that markedly alters their hydrophilic–hydrophobic balance and molecular shape. This represents an important key point at the mesoscopic level linking local properties and metabolism with lateral and transverse membrane structuring in a bio-electro-mechano-chemical cross-talk. The reduction of dimensionality at the interface [59] brings in the capacity for vectorial amplification for molecular transduction. Such cross-talk begins at the level of enzymatic catalysis whereby chemical structure is modified. In turn, this introduces the basic physico-chemical problem that is related to favorable or unfavorable tendencies for coexistence of GSLs having marked differences in their surface properties within the restricted structural region in which biocatalysis is taking place.

1.2.1. The hydrocarbon moiety region

The relative length of the hydrocarbon chains of the Cer moiety introduces marked differences in their membrane behavior which should caution on the frequent use of short-chain Cers to infer on their cellular behavior [60,61]. The presence of hydroxylated or non-hydroxylated fatty acyl moieties in long-chain Cers further induces variations of molecular packing areas and surface compressibility [62]. However, cerebrosides containing long chain mostly saturated fatty acids that are either hydroxylated (phrenosin) or non-hydroxylated (kerasin) show almost identical surface pressure-area and surface potential-area isotherms of the solid condensed type [63–66]. This actually means that the carbohydrate residue in cerebrosides partially counteracts the surface effect induced by acyl-chain hydroxylation in the Cer moiety. The fundamental message in this case is that two specific and defined changes of chemical structure can balance and compensate each other when transduced to the hierarchically higher level of the supramolecular organization. (for more examples and discussion of combined influences see refs [13,40,43,44]). The lack of an amide-linked fatty acyl chain substituent in sphingosine introduces a marked liquid-expanded character due to at least four factors: the absence of oligosaccharide chain, the presence of a positively charged free amino group, hydration/hydrogen bonding network differences at the interface, and weakening of Van der Waals forces between hydrocarbon tails [65,67,68]. In de-acetyl-lyso GM1 the simultaneous absence of both the amide-linked fatty acyl moiety and the N-acetyl group of neuraminic acid, compared to ganglioside GM1, introduces internal charge compensation at neutral pH and one additional net positive charge at the interface causing a decrease of the dipole potential density and increase of the surface stability [65,67]. De-acetylation of the sialic acid residue has a stronger influence on the hydrocarbon chain tilt angle than removal of the ganglioside fatty acyl chain [69]. These are just a few simple, but compelling, evidences pointing out some profound errors that can be made when interpreting the general surface behavior of GSLs on the basis of properties of individual moieties.

Advances in the preparation of some semi-synthetic relatively simple GSLs and improved purification procedures for species with defined sphingoid moieties allowed to better characterize the influence of hydrocarbon chains on their molecular organization and phase state. LacCer containing different saturated fatty acyl chains shows relatively high temperature- and surface pressure-induced phase transitions [70], compared to related sphingoid- and glycerol-based lipids, confirming earlier findings regarding GSLs in general with respect to phospholipids with similar hydrocarbon chains (see [44] for further refs.). LacCer monolayers show less in-plane elasticity than those formed by SM but more than that of films of GalCer [70]. The disaccharide head group only marginally disrupts gel phase packing and, in coincidence with previous reports [43], orients more perpendicular than parallel to the interface. Introducing cis double bonds in the LacCer fatty acyl chains markedly lowers the high thermotropic and pressure-induced transitions, with the greater reduction occurring when cis double bonds are located near the middle of the acyl chains [70].

1.2.2. The oligosaccharide region

Specific glycosylating enzymes and glycosidases established divertion of biosynthesizing and degrading pathways of GSLs depending on the oligosaccharide chain complexity [7,8]. For brain neutral GSLs and gangliosides, the oligosaccharide chain complexity develops from stepwise enzymatic addition of carbohydrate residues to the Cer precursor; this maintains a same, though heterogeneous, composition of the hydrocarbon moiety in the different members of a specific biosynthetic pathway [7,8]. The α- or β-glycosidically linked carbohydrates form a neutral or negatively charged oligosaccharide chain protruding from the membrane interface into the aqueous medium [13,44] and contain a plethora of hydroxyl groups mediating stereospecific recognition to ions, lectins, toxins, enzymes, antibodies and other macromolecules [1,14]. Variations from α- to β-glycosidic linkages induce marked changes of molecular packing, phase state, surface electrostatics and membrane topology [44,57,67,71]. Since more than one surface parameter is simultaneously affected the combined outcome is complex and difficult to predict based only on the presence or absence of defined moieties.

A spontaneous chemical alteration in the oligosaccharide chain of possible physiological significance is ganglioside lactonization [72,73] by which both the sialic acid charge and oligosaccharide conformation are simultaneously and reversibly modified depending on pH within the interfacial physiological range. This causes marked polar head group-dependent modifications of the sphingolipid molecular packing and dipole potential, further transduced to different interactions in mixed interfaces with phospholipids [31,74] without requiring slow biochemical alterations of the membrane composition. The presence or absence of the oligosaccharide chain (neutral or protonated at acid pH) in several uncharged sphingolipids induces a difference within about ± 0.2 nm^2 in the limiting area/molecule [43,67]. This is due to the preferred orientation of the carbohydrate residues in the polar head groups mostly perpendicular to the interface as first proposed [65], a fact that continues to be apparently "rediscovered" (actually confirmed) using several methodologies such as electrophoresis of ganglioside-containing bilayer vesicles [75], AFM, synchrotron grazing incidence X-ray diffraction and reflectivity in planar interfaces [76,77] neutron reflectivity [78], and molecular dynamics simulation [79]. The orientation of the GSLs polar head group relative to the bilayer surface is not significantly influenced by chain length but there are some differences induced by its hydroxylation. The orientation of the carbohydrate residues is sensitive to the lipid cross-sectional area [43,57,71] and to the distance of the carbohydrates from the surface which may influence the conformation and orientation of the polar head group [80]. The orientational order in the polar head group plane can extend at long range when the GSLs are incorporated into phospholipid bilayers in the liquid–crystalline state, for example, the single galactose residue of GalCer (which is proximal to the bilayer interface) and the terminal galactose in ganglioside GM1 (which is more than 1 nm away from the interface at close packing) have comparable average orientation and fluctuations about the bilayer normal [80].

Another major factor influencing membrane organization and dipole potential density in general, and that of GSLs in particular, is the long-range water structure in the bulk phase. Thus, hydrophilic solutes that modify entropy through changes of the water structural order greatly affect the surface organization [50,81]. A considerable amount of non-freezable water is associated to the polar head group of GSLs [82]. About 10 water molecules per lipid are so strongly perturbed by GalCer that no longer undergo the ice–liquid transition while each ganglioside oligosaccharide chain affects more than 60 water molecules in at least two, possibly more, hydration layers [83,84]. Interaction of acidic GSLs with proteins have also pointed out important influences of hydration–dehydration processes on the intermolecular organization [85–87]. Studies with solvatochromic probes showed that the interfacial micropolarity becomes increasingly polar in bilayers containing GSLs, compared to pure phospholipids [19,88]. These early findings were subsequently confirmed by studying the generalized polarization of the probe laurdan that indicated a more hydrated interface both in the gel and liquid–crystalline phase states of GSLs which makes the probe insensitive, and thus unreliable, for determining phase transitions or coexistence in systems containing these lipids [89]. When the two-dimensional order of the more complex GSLs is increased by compression, release of water molecules into the bulk phase occurs by coalescence of the GSLs hydration shell [50,86] which favorably increase the system's entropy in spite of the unfavorable increase of the lipid surface order due to closer intermolecular packing. However, in the case of GSLs having relatively small polar head groups, the increase of molecular order acquired by close packing is entropically unfavorable because it cannot be compensated by release of enough water molecules from the polar head group hydration shell [50,86].

Within the integrative context set out in the introduction, these are just some hallmarks of the many examples of amplified consequences brought about by thermodynamic-structural compensations due to relatively small changes of the chemical structure of GSLs. The marked dependence of the interaction energies on the molecular packing, surface electrostatics and hydration further translate into different phase states and interfacial curvature according to the GSLs polar head group (see below).

1.2.3. Molecular self-miscibility and inherent phase state

In the ganglio-series of GSLs, the compression-free energy (the two-dimensional work required to bring together the molecules from the liquid-expanded state to their closest packing) increases in approximately linear fashion per added carbohydrate but with different slopes, for neutral GSLs and for gangliosides [90]. This indicates that it is increasingly more difficult to closely pack GSLs as they contain more complex oligosaccharide chains due to steric hindrance, dipole moment and electrostatic charge repulsion, hydration–dehydration effects mediated by the complexity of the polar head group [44]. As a consequence, in a surface region where two different GSLs may coexist, the likelihood for lateral packing defects in the surface lattice increases with the polar head group

complexity. When the oligosaccharide chain of one of the two GSLs in a binary mixture is either relatively large or small compared to the other, surface packing distortions and "molecular cavity" effects can occur whereby some molecules become "hidden" in terms of molecular area contribution, while affecting independently the surface electrostatics and lattice topography [31,91,92], an effect recently confirmed using AFM [77]. It should be pointed out that the concept of "miscibility" can be quite ambiguous and misleading if not clearly focused on the particular scale range (see above) to which it is applied [90]. For example, at the molecular level, intermolecular immiscibility in binary monolayers (with or without macroscopic phase domain separation on the μm scale range) can be inferred by additive variation of mean molecular area and dipole potential density as a function of the film composition, together with composition-invariant collapse pressures [31,90,93,94]. In binary or ternary systems lateral immiscibility can be observed on the μm scale range by the presence of segregated phase or compositional domains [92,95–99]. When the system is rather complex regarding composition or surface topography, the emergence of lateral thermodynamic tensions and interfacial energy terms eliminate cooperativity at the local level. This has the consequence of the film showing additive behavior and smooth compression isotherms that could be erroneously interpreted as corresponding to a homogeneously mixed surface while it actually exhibits a richly featured surface topography with coexistence of immiscible domains of different composition and phase state [92,97–100]. Nevertheless, the existence of microheterogeneity on the μm scale range in the surface topography implies local interactions leading to favorable or unfavorable intermolecular mixing of the different components along the lateral plane. In monolayers with a complex composition prepared with the whole myelin membrane, ganglioside GM1 and GalCer localize in segregated surface domains [98].

Similar to phospholipids for which the thermotropic behavior of bilayer vesicles is correlated to variations of the molecular packing in monolayers [93,101], GSLs can exhibit all types of isothermal, surface-pressure induced, two-dimensional phase states depending on temperature and the type of oligosaccharide chains [86,87]. In the temperature range between 5 and 65 °C, the more complex gangliosides in monolayers remain in liquid-expanded state and no pressure-dependent transition is found. The increased thermal energy and configurational entropy contributions derived from the hydrocarbon and oligosaccharide moieties cause a decrease of the cohesive dispersion energy and the molecular packing areas increase progressively with the rise in temperature [43,87]. Neutral or acidic GSLs with short polar head groups (GalCer, Sulf) or with oligosaccharide chains of intermediate complexity (Gg3Cer,Gg4Cer, GM3, GM2) clearly show isothermal surface pressure-induced transitions [86]. Even moderate fluctuations of the surface pressure (5–10 mN/m about the average value) can induce isothermal phase changes involving considerable variation of the intermolecular packing as indicated by defined changes of slope in the corresponding molecular area-temperature isobars [86,87]. Variations of the surface thermodynamics due to changes in hydration of the oligosaccharide chains are implicated in determining the lateral organization and phase state of the interface [50]. Again, these cross-related and mutually dependent factors represent structure-specific molecular codes determining the surface organization in an integrated manner. In aqueous dispersions, the structural and thermal properties of totally synthetic C18-sphingosine (C16:0)-GalCer and GlcCer show complex but quite similar bilayer thermal phase behavior. This indicates that the precise isomeric structure of the linked hexose plays an insignificant role in regulating the polymorphism of hydrated cerebrosides [102], which is in agreement with the similar monolayer behavior and bulk phase transitions described for natural GalCer and GlcCer [44,65]. In addition, the thermal behavior and bilayer phase formation of synthetic GalCer and GlcCer is not changed by the heterogeneity of the sphingosine base of natural and partially synthetic cerebrosides [102]. The presence of a $-SO_3$ group in C16:0 cerebroside-sulfate results in a marked decrease of melting temperature (T_m) compared to GalCer and unusual hydration effects and surface charge-induced alterations of the hydrocarbon chain melting. The bulk T_m of C16-cerebroside-sulfate reaches 49 °C at fully hydration [103], a value close to the 50 °C previously reported for bovine brain Sulf in excess water [44,104,105].

The more simple GSLs have mean molecular areas and polar head group sizes similar to those of phospahtidylcholines [43]. However, the values of the bulk T_m are 20–40 °C higher compared to phospholipids with similar hydrocarbon chain length and unsaturation [40,93,104,106] and this also occurs for SM [107,108]. Similar to the monolayer behavior, chemical or conformational differences in the oligosaccharide chain of GSLs introduce marked variations of the bulk T_m [87,104]. A network of inter- and intra-molecular H-bonds involving the carbohydrates, the Cer hydroxyl as well as the amide groups, together with the polar head group-bound water influences the interfacial properties and phase behavior of GSLs. An increase in the number of carbohydrates in the oligosaccharide chain or the presence of hydroxyl groups in the amide-linked fatty acyl chains bring about further capacity for hydrogen bonding which could strengthen molecular cohesion. But the compensation and amplification resulting from different intermolecular effects that translate local structure on the nanometer scale to the supramolecular range are not as straightforward as reductionism would dictate. In spite of the negative charge and the lack of hydroxylated fatty acyl chains in synthetic N-palmitoyl-Sulf its T_m and transition enthalpy are higher than those of N-palmitoyl-SM [109]. However, the T_m of sulphatide containing hydroxylated fatty acids is also higher than that of the non-hydroxylated form. These variations have been interpreted as due to the simultaneous and mutually influenced participation of the hydroxyl groups of the fatty acyl chain and the galactose residue in the surface network of hydrogen bonding [109–111].

As the number of carbohydrates (and also the possibilities for hydrogen bonding) increase in the oligosaccharide chain, a decrease of T_m and transition enthalpy are observed instead of an increase, indicating diminished intermolecular cohesion

[44,87,104]. Except for samples at relatively low hydration, another powerful factor is obviously overriding the establishment of long-lived network of hydrogen bonding between the GSLs molecules. When the latter are properly hydrated, extensive H-bonding to water increases the size of the lipid hydration shell [50,82–84] and interferes with carbohydrate–carbohydrate interactions. This impediment can be further emphasized when the relative orientation of donor and acceptor groups at the surface are critical [43,112]. The temperature required for reaching a fully liquid-expanded state in monolayers of GSLs decreases as the oligosaccharide chain becomes more complex and hydrated [86] and this is similar to what is found for the gel-to-liquid crystalline bulk T_m in aqueous dispersion [104]. The correlation of the T_m and enthalpy with the lateral intermolecular packing [44], itself highly dependent on the surface pressure, actually means that fluctuations of the latter are transduced to surface phase changes.

The temperature-driven gel–liquid crystalline transition properties of a lipid originate in the relative changes of the distribution of molecules in configurational energy states that correspond to the gel and liquid–crystalline phases. In essence, this is what constrains the intermolecular organization and topographical distribution of coexisting phase domains [46,113]. The thermodynamic parameters corresponding to the calorimetrically determined excess heat capacity–temperature function reflect the variation of the size and number of coexisting gel and liquid crystalline domains that are in dynamic equilibrium during the phase transition [114,115]. In relatively complex systems, statistical thermodynamic modeling is limited because several contributions to the partition function are unknown or their estimation is uncertain. An operational treatment that obtains the partition function directly from experimental excess heat capacity–temperature functions [114,115] allows calculation of thermodynamic cluster domain averages and surface densities, gel–liquid crystalline fractional boundaries, probability distribution functions and fluctuations underlying each individual T_m without requiring model assumptions for molecular configurations. Since any inference about the latter is absent, the size distribution functions correspond only to "thermodynamically correlated" molecular changes that may not have a simple or direct topographical correspondence. For the thermotropic behavior of GSLs in self-assembled bulk aqueous dispersions, the size of the calorimetric cooperative unit and of the thermodynamic clusters vary according to the GSLs oligosaccharide chain. This variations follows closely the trend of the resultant polar head group dipole moment perpendicular to the interface [43,44,65]. As noted before [57], the overall molecular dipole moment vector of GSLs, normal to the interface, decreases as the oligosaccharide chain becomes more complex until it acquires a minimum value when it contains about three to four carbohydrates and then increases again for the more complex gangliosides [44]. The variation of the size of the average gel cluster with the local polar head group dipole moment strongly suggests that, similar to systems containing phospholipids [96] or Cer [116], dipole moment repulsion in relation to line tension is an important determinant for establishing the cluster size and to eventually induce long-range domain super-structuring at the mesoscopic level [99,116]. On the other hand, several factors derived from different thermodynamic tensions are involved in establishing the surface domain topography. As the GSLs polar head group becomes more complex, the differences in the relative sizes of the oligosaccharide chain and the hydrocarbon moiety introduce curvature tensions in the self-assembled structure (see below and [117,118]. This affects concomitantly the overall topology and the lateral phase state as indicated by the fact that curvature tensions cause that the T_m for the bulk phase transition of GSLs aqueous dispersions is lower than the temperature at which a fully liquid-expanded state is acquired in the flat two-dimensional monolayers [44,87]. Adaptation of the statistical thermodynamic approach for the isobaric bilayer phase transitions to the surface pressure-induced liquid-expanded to condensed isothermal transition in monolayers indicates that the sizes of thermodynamically correlated phase domain when GSLs are constrained to remain in monolayers (zero curvature) are smaller and similar for all GSLs, independently on their oligosaccharide chain; the different behavior revealed for the phase changes in monolayers and bilayers emphasizes the important role of tensions derived from curvature and overall topology in determining the thermodynamic correlations controlling the surface phase structure.

1.3. Surface topography: projection of molecular shape and miscibility of GSLs into formation of coexisting membrane domains

1.3.1. Segregated membrane domains, the beginning

In most compositionally complex polymorphic liquid–crystalline systems such as those constituted by biomembranes, it is thermodynamically inescapable that the different unbalanced tensions result in lateral or transverse segregation of components. As elegantly expressed elsewhere [119], membranes can be considered loosely ordered microheterogenous structures of evanescent associations. These are in a metastable far-from-equilibrium state in which thermodynamic, geometrical and viscoelastic tensions determine the spontaneous emergence, shape and composition of segregated domains as transient patches of differentiated membrane structuring in composition and/or phase state.

The idea of compositional or phase domain segregation, well known to membrane biophysicist for over 40 years, simply reflects the basic physico-chemical phenomena of eliminating or reducing local tensions derived from thermodynamic incompatibility among different molecules by establishing lateral or transverse immiscibility, a symmetry-breaking event with long-range supramolecular consequences. This concept, with its inherent dynamics and metastability, initially emerged well back in time and was derived from three lines of research on artificial model membrane experiments: (a) the existence of phospholipid phase transitions with isothermal lateral phase separation and coexistence, driven by changes of surface pressure, composition and interactions [120]; (b) protein-induced lipid phase separation with selective lateral mobility restrictions of the boundary lipid hydrocarbon moiety along the

membrane plane [121]; (c) lipid hydrocarbon chain interdigitation, hemibilayer coupling, and transverse propagation of phase-mediated defects across the lipid bilayer [122,123]. Another important finding in simple binary systems was the description of the in-plane coexistence of two liquid phases with different organization, the liquid-ordered and liquid-disordered phases, in which the presence of cholesterol (CHOL) is important [124–126]. The concept of segregated domains, and their structural (or eventually functional) significance, remained largely ignored in biochemistry and cell biology for many years while a large body of biophysical studies continued to accumulate disclosing many of their properties in well defined and controlled lipid–protein systems; liquid phase coexistence in whole natural membrane surfaces with a complex composition was demonstrated later on [97,127,128]. Along the last decade, the old concept of lipid- and protein-induced compositional immiscibility at the membrane surface was essentially "rediscovered", regained its deserved originality, and was renamed with the somehow more appealing (but also more misleading) name of "rafts" and became explosively popular. After so many years of efforts done by basic membrane biophysicists, it may even appear as amusing that some of the more popular conceptions adopted in the field of cell membrane biology today have originally derived from frequently called "biologically irrelevant" studies done with synthetic lipids (some of them not even present in natural membranes) and artificially simulated biointerfaces.

Cell membrane domains have been defined operationally on the basis of methods used to detect or isolate them, and according to technical preferences. So-called "rafts" and many other putative domains [detergent insoluble glycolipids (DIGs), detergent insoluble membranes (DIMs), detergent-resistant membranes (DRMs), detergent insoluble glycosphingolipid-enriched microdomains (DIGEM)] are generally used as synonyms despite their largely different origin and conceptual meaning but it is important that they not be confused [124,129]. Actually, most segregated domains reasonably well characterized in terms of structure and biophysical properties remain without proven function while some presumably well-described membrane function could not yet be associated to defined domains [119]. Also, from the rather restricted and defined components initially conceived as forming these domains, their composition became increasingly complex over the years to include a rather large variety of lipids and proteins. Some major advances were made in understanding the effects of Triton X-100 on membrane solubilization [124,130–133]. These experiments indicated that the detergent disorders the liquid-disordered phase, and orders the liquid-ordered one, in the canonical "raft mixture" of equimolar proportions of POPC, SM and CHOL by partitioning selectively into the more disordered phase and this effect is accentuated by cooling, actually promoting liquid-ordered domain formation [131]. The detergent can even induce domain formation by cooling from an initially homogeneous mixtures; also, the composition of detergent-resistant membranes isolated from cells by subcellular fractionation can be markedly altered and is finely dependent on both the temperature treatment and the detergent concentration [134,135]. The small transition enthalpy between the liquid-disordered and liquid-ordered phases in the ternary "raft mixture" makes domain formation or disintegration very sensitive to small variations induced by temperature, composition and surface pressure [132]. It was recently emphasized that, considering results of studies in biological as well as model membranes, the existence of relatively large and long-lived lipid raft domains in cell membranes is unlikely [130].

1.3.2. Glycosphingolipid-enriched domains: the assumptions

It would have indeed been surprising if GSLs, with their multimodal effects in biomembrane behavior, could have escaped the ongoing fashion. However, several misconceptions exist regarding the participation of these lipids in membrane domains. Comparison of the distribution of SM-rich domains with ganglioside GM1-rich domains in the plasma membrane of T-cells showed lack of colocalization and spatial heterogeneity on a submicrometer scale range [136]. In mast cells, clusters of IgE receptors and antigen Thy-1 are very small and ganglioside GM1 is rarely clustered at all; when induced to cross-link externally, membrane components redistribute with independent behavior into membrane domains that can exceed 500 nm diameter and that appear stabilized by physical interactions [134]. Recently, it was clearly shown that cold detergent treatment caused ganglioside solubility and alteration of their tissue distribution leading to redistribution between gray and white matter in mice brain slices [137]. Understanding the molecular factors determining the presence and miscibility of GSLs in interfacial lipid domains segregated from other membrane components has actually been an early concern [19,138,139]. The biophysically based assumptions by which these lipids would be laterally enriched in segregated membrane domains have been the following. (i) glycolipid tendency to self-associate and phase separate from glycero phospholipids. This would be presumably driven by the hydrogen-bonding capacity of the sphingolipid backbone region, and direct H-bonding between carbohydrate residues [140]. (ii) Preferential interactions between sphingolipids and CHOL compared with PC-CHOL, not weakened by glycero-phospholipids; this would be a reason for their formation and stability to cold detergent extraction [141]. Detergent insolubility would reside in properties of LO-type phases, assumed to be strengthened by the presence of lipids with high T_m and by GSLs H-bonding capacity [128,142]. (iii) Specific oligosaccharide chain of GSLs would determine selective enrichment in domains of different composition [143–145]. It is worth to briefly analyze one by one some experimentally proven facts regarding these popular assumptions.

1.3.3. Do GSLs spontaneously mix or closely pack among themselves?

Close molecular packing among single GSLs is thermodynamically unfavorable at the local level and they become expanded in proportion to the complexity of the oligosaccharide chain [43,90]. The type and relative complexity of the polar head group of a natural species in a binary sphingolipid mixture are of paramount importance for establishing the

molecular miscibility properties, the type, and the particular details of the interactions [90]. Interactions among neutral GSLs, and between these lipids and Cer, occur with thermodynamically unfavorable expansions (positive excess free energy of mixing) of the mean molecular area and increases of the resultant molecular dipole perpendicular to the interface (hyperpolarization). The changes are more marked when the oligosaccharide chain of the GSLs is more complex; the intermolecular incompatibility is further emphasized when the binary mixture is constituted by two neutral GSLs with similar polar head group sizes containing an increasing number of carbohydrate residues. On the other hand, interactions of Cer with gangliosides are characterized by thermodynamically favorable condensation of the mean molecular area (reflecting increased intermolecular cohesion) and favorable matching of the resultant molecular dipoles leading to surface depolarization, in proportion to the complexity of the ganglioside polar head group. Binary mixtures of LacCer or Gg4Cer with any of the gangliosides and all mixtures between different gangliosides reveal immiscible behavior on the molecular scale range. Based on local average interactions, it was concluded that it is thermodynamically unfavorable for GSLs with polar head groups of similar size to undergo mixing between them [43].

1.3.4. Do GSLs tend to self-demix and phase separate from phospholipids?

All GSLs and Cer were found to spontaneously mix non-ideally on the molecular scale range with different phospholipids in monomolecular films [43,90,146]. In bulk dispersions temperature-composition phase diagram of binary systems of DPPC and neutral GSLs with different oligosaccharide chains show marked gel-phase and some liquid-phase immiscibility in which isothermal melting of the pure phospholipid is observed over a rather wide range of composition; on the other hand, no isothermal melting corresponding to laterally segregated pure GSLs is found over the whole phase diagram and not even on the GSL-rich side of the composition range. This behavior was indicated many years ago [139] showing that GSLs do not tend to form a separate phase by themselves but actually become segregated in GSLs-enriched, but mixed, clusters with phospholipids [19,139]; this is driven by the spontaneous phase separation of isothermal melting pure phospholipid domains that exclude GSLs [13]. In fact, interactions among neutral GSLs are too short-range (mostly different types of H-bonding) to constitute a driving potential for long-lived GSLs clustering and the negative charges on gangliosides preclude their close association [13,90]. On the other hand, intermolecular H-bonding (clearly demonstrated only for the more condensed GSLs such as cerebrosides and Sulf, [110,147,148] after or during domain formation may help stabilize specific GSLs within a localized membrane region [144].

Mixtures of Cer with gangliosides are thermodynamically and electrostatically favorable; furthermore, they can lead to geometrical–thermodynamic compensation of curvature stress and favorable increase of intermolecular cohesion, narrowly dependent on the relative proportions, that can drive topological restructuring [91]. However, an oligosaccharide chain length of two or more neutral carbohydrates or addition of a negatively charged sialosyl residues constitutes a critical limit beyond which a marked difference in behavior occurs, with establishment of molecular immiscibility. Interactions among GSLs and with other lipids or proteins [13,44,149] may contribute to topologically stabilize sphingolipids in a bilayer structure (see below) so that gangliosides can be retained in relatively large proportions in domains containing different types of gangliosides [144,150,151]; this may even exceed the critical amounts at which their intrinsic geometry would spontaneously induce membrane micellization [118,152]. On the other hand, relative GSLs enrichment in the liquid ordered phase does not vary in any consistent manner with the type of oligosaccharide chain [153]. Taken together, all the available results obtained so far, do not support the proposal that the type of oligosaccharide chain determines a selective partitioning in different domain populations.

The various sphingolipid precursor–product relationships and specific enzymatic steps for GSLs and ganglioside biosynthesis have been well characterized [7,8]. Direct extrapolation of results obtained with a simplified interfacial system, albeit well controlled at the molecular packing level, to more complex mixtures is not straightforward, let alone to natural membranes containing several other lipids and proteins whose precise molecular interactions are not known. Nevertheless, it is of suggestive and significant biochemical importance that some natural GSLs mixtures constituting key diverting points for specific biosynthetic routes show a markedly different average behavior. The changes of interactions brought about by the addition of a single sialosyl residue to LacCer to form ganglioside GM3, or another one to the latter to form ganglioside GD3 (three GSLs located at key branching points of series-specific biosynthetic pathways), generates local molecular immiscibility [90], thermodynamically forcing the product out of the entourage of the parent compound. Similarly, the presence of each successive carbohydrate residue leads to further unfavorable molecular mixing thus driving increasing lateral segregation of the more complex species. It is possible that the basic tendencies of GSLs to undergo surface mixing-demixing processes may not take place in a complex natural membrane surface. However, in this case, other biochemical or biophysical events so far undisclosed would need to occur and be explained at the molecular level in order to account for differences in behavior that could override the intrinsic molecular thermodynamic tendencies of GSLs and ganglioside to segregate.

1.3.5. Is the linkage region of sphingolipids responsible for self-segregation?

The main structural characteristic that distinguishes all sphingolipids from glycerolipids is the “linkage backbone” connecting the head group moiety to the hydrocarbon chains. In sphingolipids the presence of amide and hydroxyl groups with both H-bonding donor and acceptor capacity has been shown to occur among clustered GSLs in bilayers [110] and thought to be an intermolecular linking element to promote self-clustering. However, recent studies designed to

specifically test participation of the sphingolipid backbone in their association has indicated that intermolecular H-bonding in the linkage region cannot be a driving force for their selective segregation into phase-separated domains. Contrary to frequent assumptions, affinity of sphingolipids toward glycerolipids is greater than affinity toward themselves, and self-association of sphingolipids is in fact disfavored [154]. These results are coincident with early conclusions [65] on sphingolipid unfavorable self-interactions, but favorable mixing with phospholipids, on the basis of calorimetric and monolayer studies [13,90]. Also, the assumption that direct interactions among carbohydrates in the polar head groups of GSLs could cause self-association has received no experimental support; on the contrary, it was shown that the presence of a lactose moiety in the polar head group works against homo-association and actually weakens intermolecular interactions by reducing packing efficiency, contributing "bulkiness" but not "stickiness" [154], as indicated many years ago by the progressive increase of the GSLs mean molecular area and decrease of their T_m as the oligosaccharide chain becomes more complex [104,146]. It is necessary to emphasize that the point is not if some type of sphingolipid can be found in segregated enriched domains (an established fact), or if topographical domains occur in biomembranes (surface microheterogeneity is also well proven) but what is the initial driving potential at the local molecular level for spontaneous sphingolipid segregation in domains and how thermodynamically stable or long-lived the associations might occur the dynamic interfacial conditions (let alone introducing further harsh conditions for their putative isolation). So far, the reason for GSLs enrichment in segregated domains does not appear to be accounted for by their individual molecular properties or chemical structure.

1.3.6. Is the presence of GSLs a major factor for detergent insolubility?

In systems of PC/SM, the higher the SM proportion, the less detergent required for solubilization [133,155]. Thus gel phase state or liquid-ordered phases, *per se*, do not imply insolubility. For PCs (T_m's in the range of −10 to 65 °C), and for pure SM (T_m~40 °C), gel phase state required less detergent for solubilization, compared to liquid-crystalline phase [156,157]. IR and EPR of PC/SM and PC/SM/CHOL showed H-bonding between amide carbonyl of SM and hydroxyl of CHOL but the sterol contributes more to detergent insolubility at 37 °C than at 4 °C; PC weakens the interactions between SM and CHOL in the liquid ordered phase and facilitates detergent solubility [158,159]; the mixtures are detergent-solubilized at least equally, but generally more easily at 4 °C than at 37 °C. Sphingolipids by themselves (and specially GSLs) do not hinder but generally increase propensity to solubilization [133].

1.3.7. Are gangliosides preferentially segregated into selective phase domains?

Several reports, mostly using ganglioside GM1 in reconstituted biomembrane models indicated that ganglioside GM1 affects domain features. An early report on these effects, using far-field epifluorescence and near-field scanning optical microscopies [160], showed that addition of a very small percentage (<1 mol%) of GM1 to a monolayer of DPPC had significant effects on the surface structure leading to sprouting and growth of thin buds from the domain boundaries, implying increases of the line tension due to the allowed decrease of the dipole moment density by loosening of molecular packing in the domains in order to reduce dipolar repulsion within the domains; this is in keeping with the interfacial depolarizing properties and molecular packing expansion capacity of GM1 [43]. Most of the morphological studies available performed with epifluorescence or AFM done with different lipid mixtures containing GM1 essentially describe similar results to those first reported. The alterations consisted in distribution of GM1 in the phospholipid matrix, with features of the coexisting domains that depended on the relative lipid composition and lateral surface pressure [161–163]. CHOL is not miscible with synthetic N-palmitoyl-GalCer in the gel phase but becomes miscible in the liquid–crystalline phase [164]. An important effect of the hydrocarbon portion is deduced since about 34 mol% CHOL is required to eliminate the liquid–crystalline transition of brain GalCer compared to 50 mol% to abolish that of N-palmitoyl-GalCer [164]; in ternary mixtures, a homogeneously mixed phase of DPPC and CHOL saturated with 20–23 mol% N-palmitoyl-GalCer coexists with an excess cerebroside phase [164,165]. In binary systems, DPPC is unevenly distributed among gel and liquid–crystalline phases depending on the type of GSLs, composition and temperature [139]. Marker proteins that bind to GSLs showed that GM1 and asialo-GM1 at low mole fractions are localized preferentially in gel phase domains of binary phospholipid mixtures while at higher proportions are also found in the fluid-phase due to preferential interactions among the hydrocarbon moieties causing differential domain partitioning [166–169]. Calorimetric studies of ternary SM/ganglioside GM1/CHOL systems with variable proportions of ganglioside and CHOL displayed lateral phase separation addressable either to SM/CHOL or to SM/GM1 mixtures on increasing the sterol or ganglioside contents, respectively [150].

Scanning AFM of Langmuir–Blodgett monolayers formed with a mixture of SM/palmitoyloleoylPC/CHOL/GM1 showed that the ganglioside is preferentially segregated in an ordered lipid matrix [170]. An important issue that was little explored is the mobility of membrane components inside and outside segregated domains. Only slight differences were found for the mobility of fluorescent hydrocarbon tail-labeled GM1 or polar head group-labeled asialo-GM1 in submicrometer fluid domains while mobility was severely attenuated in gel phase DPPC, although mobility around domain boundaries of the gel phase resembled that in the fluid domains [171]. Biologically relevant GM1 concentrations lead to submicron-sized domains, as detected by AFM, in CHOL-rich liquid-ordered phase [172]; in phospholipid–CHOL–SM mixtures studied by fluorescence microscopy GM1 was found again enriched in the more ordered domains reportedly resistant to cold detergent extraction [128], a finding contrary to that of others [133]. It was recognized that many fluorescent probes used to examine the domain

segregation phenomena are ill-suited for obtaining valid conclusions [151], and that most GSLs, and moreover gangliosides, actually favor cold detergent solubilization in model systems [133]. Synchrotron grazing incidence X-ray diffraction and reflectivity studies [76] found no evidence for segregation of ganglioside GM1 into domains when incorporated in mixed monolayers with DPPE. In natural cell membranes the interaction of gangliosides with external ligands can lead to patching and capping phenomena [166–168,173]; however, although probably related to binding and cross-linking, surface clustering of fluorescent gangliosides [174] or cholera toxin [175] does not occur thus questioning direct binding as the driving mechanism. On the other hand, it was recently shown that binding of cholera toxin to ganglioside GM1, as a minor component of a more complex lipid mixture constituted by DOPC, DOPG, SM and CHOL initially homogeneous, induced phase separation into coexistent liquid-ordered and liquid-disordered membrane domains; in addition, a long range implication of the ganglioside–toxin binding on the surface structure was shown since the phase separation caused redistribution of a transmembrane peptide [176]. In monolayers formed with the whole myelin membrane, specific immunofluorescence labeling of GalCer and fluorescent cholera-toxin labeling of ganglioside GM1 indicate that the two sphingolipids localize in different surface domains. In this compositionally complex membrane surface exhibiting liquid–liquid phase coexistence [97,98,177], the ganglioside labeling is localized in liquid-expanded regions, not in the liquid-ordered domains, together with specific labeling of major myelin proteins while immunolabeling of GalCer is found in the liquid-ordered domains co-localizing with specific markers for CHOL and phosphatidylserine [98].

1.4. Beyond the membrane interface: glycosphingolipid as modulators of structural topology, bilayer recombination and surface biocatalysis

1.4.1. Escape into third dimension, tension relaxation by thickness and curvature

The effects described in the previous section clearly represent an extraordinary capacity for information transduction by GSLs that is conveyed laterally on the surface lattice but this is also done transversally across the membrane plane. The different length and/or average orientation of the oligosaccharide chains present in GSLs existing in various phase states clearly suggest a relationship between the type of GSL polar head group, the lateral topography and the interfacial thickness. Unlike epifluorescence that distinguishes the differential partitioning of fluorescent probes [95,96,142,151,178], Brewster Angle Microscopy (BAM) derives contrast from differences in the optical properties of thin films. Through a quantitative measurement of the light reflected at the interface, BAM also allows to calculate the relative change in thickness of defined surface regions [95,178]. Sphingolipids follow different regimes of variation of reflectance versus surface pressure according to their phase state. Cer and GalCer, with polar head groups containing only a hydroxyl group or a single galactose residue, undergo small changes of reflectance indicating that the film thickness remains, on average, rather constant during compression [92]. These results are in agreement with previous ones showing that these lipids form very condensed films, with small changes of intermolecular packing and polar head group orientation under compression [37,44,179]. The topographic appearance by BAM of films of GalCer and Cer at low surface pressure is dominated by the presence of highly mobile, rigid cluster domains, with irregular boundaries, coexisting with gas phase. As the film is compressed, the clusters fill out the optical field and the surface acquires a more homogeneous appearance; asialo-GM1 (Gg4Cer) and GM1 have large polar head groups, a liquid-expanded interface, and BAM images reveal a rather homogeneous surface with the reflectance showing notorious changes [92]. This agrees with previously published measurements of surface (dipole) potential that indicated reorientation of the oligosaccharide chain of both lipids depending on the surface pressure [13,35,43,44,179]. The perpendicular dipole moment of Cer increases as the molecules become more closely packed due to the surface pressure-induced stretching of the hydrocarbon chains as they become condensed. It is well known that the positive end of the dipole moment of aliphatic chains points up to the air side of the monolayer; for a saturated chain of 16–18 carbons the magnitude of the dipole in the condensed state amounts to a maximum of about 350 mD [180]. The maximum achieved by Cer reaches 562 mD due to the influences of some unsaturation and the hydrated hydroxyl group in the polar head. The carbohydrate residues of GSLs contribute with a resultant dipole moment generally oriented opposite to that of the hydrocarbon portion and increases with the complexity of the oligosaccharide chain [65] which brings about a decrease of the molecular dipole moment. In addition, the variations with packing of the resultant dipole moment of the GSLs indicate oligosaccharide chain reorientation into the aqueous subphase more perpendicular to the interface [43,57,65]. In good agreement with these results, the surface reflectance increases gradually with film compressions suggesting progressive thickening of the films [92].

The actual value of temperature and the heat adsorbed for establishing two-dimensional phase changes in GSLs with different oligosaccharide chains are always higher than their respective bulk T_m and transition enthalpy in bilayer vesicles. This was shown to derive from structural and thermodynamic tensions due to curvature in the bilayer vesicle, required to accommodate the increasingly conical molecular geometry of the more complex GSLs. The progressive predominance of the oligosaccharide (size, composition, charge and hydration, including the interfacial region with H-bonding capacity) with respect to the hydrocarbon portion (length, unsaturation and volume, including temperature-dependent chain isomerization) determine different local packing parameters. This establishes stringent tensional limits for the formation of structural aggregates with defined curvature. It has been previously demonstrated that the self-assembled structures of GSLs spontaneously formed in aqueous medium are in keeping with the local molecular geometry [44,117,118].

The polar head group size, protrusion and optimal area exposed to the aqueous phase in relation to the volume and length of the hydrocarbon moiety determine a molecular shape further away from a cylinder and more similar to a cone as the GSLs are more complex [47,117,118,152]. Theoretical and experimental studies have shown that neutral GSLs are compatible with a bilayer structure of increasing curvature in the order GalCer ≈ GlcCer > LacCer > Gg3-Cer > Gg4Cer. For GSLs with relatively short oligosaccharide chain the packing constraints allow their assembly in stable bilayer vesicles, with relatively low free energy per molecule and the competing factor limiting their size being the entropy of the ensemble. This imposes an upper limit for the number of lipids forming the structure that must be compatible with the entropy of the aqueous phase continuously favoring lipid aggregation and close packing in order to minimize the aqueous/hydrocarbon interface. Positive interfacial curvature [47] is favored due to the increase of area per polar head group exposed to water in relation to the hydrocarbon chain volume; on the other hand, a similar effect would occur at constant average lateral surface pressure for the more complex GSLs because of the increased molecular area required by the hydrated oligosaccharide chain [117,118]. Thus, factors that affect intermolecular packing are simultaneously transduced to curvature alterations of the interface and vice-versa, with the surface free energy of the molecule varying in correspondence; within certain limits, this may not conflict with entropy but, as the molecular geometry supports increasing stress, the structural stability will be affected with the consequence of amplifying the lateral perturbations to surface topography changes and/or topological rearrangement. Domains enriched in complex GSLs should spontaneously tend to increase curvature away from the aqueous interface. These effects will occur spontaneously if lateral pressure fluctuations drive the molecular packing areas to exceed the critical limits compatible with the interfacial curvature. Two major consequences may occur depending on how the stress is transduced by relaxation. If the fluctuation is relatively small it may be absorbed by the membrane elasticity; the periodical temporal and spatial tangential stress wave and variation of compressibility may dissipate through other factors that control surface topography such as changes of phase state and intermolecular interactions in both the polar head group and hydrocarbon regions [48,87,146,181]. If the fluctuations are larger, the membrane curvature will have to concede changes inevitably introducing tension energy costs; this may be balanced or contained up to when the membrane elasticity becomes no longer compatible with the surface stress, at which point the aggregate undergoes abrupt reorganization in its structural topology [118,182,183]. There are many experimental observations confirming early findings regarding the combined and amplified influences of the local conformation, overall hydration, charge, size and orientation of the oligosaccharide chain in determining the thermodynamic stability, shape and lateral topography of GSLs in self-assembled interfaces [43,44,152].

1.4.2. Thermodynamic–geometric compensations and topology

In systems constituted by more than one type of molecules further compensations emerge that enhance or alleviate stress than can be reflected in the overall topology. It was shown that small amounts of HI- phase-forming lipids such as specific gangliosides in binary or ternary mixtures with other lipids that spontaneously tend to form non-bilayer HII-phase cause facilitation, impairment or elimination of the HII-phase structure [35,36,38,39]. Recent studies showed the importance of mutual intermolecular thermodynamic–geometric compensations and lateral condensation among sphingolipids with different local geometries for the adoption of self-assembled structure of defined curvature, depending on their relative proportions [91], an effect also brought about by CHOL [184].

In GSLs, the sphingosine base of 18 carbons penetrates into the bilayer to a depth of only about 13–14 carbons while the amide-linked fatty acyl chain is even longer than the hydrocarbon portion of most phospholipids and can extend to a length of 20–24 carbons [185–187]. It was demonstrated that chain disparity leads to chain interdigitation with the important implication for transversal information transmission by hemi-bilayer coupling [122,123,188]. The long-chain fatty acyl residue of some GSLs extends across the lipid bilayer mid plane and penetrates substantially into the opposing monolayer [189–191]. When the proportion of asymmetric sphingolipid is increased above 30 mol% the membrane adopts a partially interdigitated structure depending on composition and temperature [111,138,190]. If the hydrocarbon chains of Cer, the basic moiety of all GSLs, are sufficiently asymmetric it can undergo chain interdigitation in phase separated Cer-enriched domain depending on the relative proportions with phospholipids [192]. This inherently implies the capacity for transverse information transduction since both halves of the bilayer become essentially coupled in those membrane-spanning regions.

Another manner for transmitting transverse information across the membrane is by topological rearrangement involving non-bilayer phases. Cer has a very small polar head group in relation to the hydrocarbon chain volume which conveys a preference for self-organizing into negative curvature structures, favoring HII-type of phases on which basis it flips-flops rapidly across the bilayer [193–195]. In addition, HII phase-like regions are key structural intermediates for inducing cell and lipid bilayer membrane fusion or fission. It was conclusively demonstrated that gangliosides and other GSLs facilitate or interfere with HII-phase formation in a manner that depends on the relative proportions of other membrane components [35,37,38,196,197]. These effects correlate with the capacity of several GSLs to affect fusion-mediated neurotransmitter release [198], cell [196,197] and bilayer vesicle fusion [38,39,199,200]. Transient structures of the HII-phase type are important intermediates involved in the hemi-fusion and whole fusion of membranes [201] that can be triggered by a variety of lipids [202–204], water soluble agents [205,206] and proteins [197,200,207,208]. In addition, thermodynamic–geometric compensations can abolish the surface stress leading to membrane reorganization and fusion when two fusogenic compounds, each of them individually facilitating HII-phase

formation, are simultaneously present in the membrane [197,198]. As a further tension-relieving effect, both ganglioside GM3 and Cer can undergo spontaneous flip-flop in bilayer lipid vesicles as a consequence of sphingomyelinase (SMase)-induced Cer on the outer leaflet [209].

At least three interrelated levels of supramolecular control of membrane interactions operate in systems containing myelin GSLs and myelin basic protein (MBP). These comprise composition-dependent variations of intermolecular packing, of surface electrostatics involving molecular dipole moment reorientation, and of the phase state. Their dynamics follows the general principles governing information exchange among micro-, meso- and macro-scales of complexity [46] by which an increase of the system's size is concomitant to slower processes and viceversa [42]. Thus, the rate and topological features of membrane phenomena taking place among bilayer vesicles, being several orders of magnitude larger in size and slower in time, compared to molecular interactions integrate and reflect simultaneously all the fluctuations at the lower level. Interactions induced by MBP between bilayer vesicles containing Sulf or GalCer, two major GSLs of myelin, involve fast membrane apposition but further changes leading to bilayer merging and whole bilayer vesicle fusion are largely arrested, this also occur for membranes containing ganglioside GM1 that is selectively enriched in myelin [38,39,199,200,210]. The interactions of MBP with Sulf cause dipole potential depolarization while hyperpolarization was found in mixtures of the protein with GalCer [211,212] and these effects are in line with the facilitation by Sulf, and impairment by GalCer, of the protein-induced membrane apposition [199,200,210]. Besides the concentration- and composition-dependent cell membrane fusion induced by gangliosides [196], it was found that these lipids affect the Ca^{2+}-dependent fusion-mediated neurotransmitter release by exocytosis of synaptic vesicles and dopamine uptake in brain synaptosomes, a process that is regulated by thermodynamic compensations due to interactions with other lipids or proteins [198,213]. As discussed above, polysialogangliosides can become laterally segregated by phospholipids into enriched domains whereby the stress generated may cause transient formation of non-bilayer phases and increased permeability [44,15,167,214,215]. Clustering of gangliosides around glycoproteins and other proteins as a regulatory mechanism underlying the capacity of gangliosides to affect the membrane structural stability is supported by spin labeling studies [216]. However, in the absence of other stabilizing interactions (see below) extensive enrichment or clustering of gangliosides would not be possible whenever their concentration should increase above 15–30 mol% since this will render the membrane unstable and force its reorganization [117,118,152].

Studies regarding GSLs–protein interactions were mostly directed to those with some direct physiological implication. These refer to the interactions of ganglioside GM1 with cholera toxin and some others involving lectins, hormones or antibodies. With few exceptions [217,218], scarce attention was directed in general to understand the molecular details of these interactions and their perturbation of the membrane structural dynamics. Recently, it was shown that upon toxin–ganglioside binding the density of the lipid layer, as revealed by neutron reflectivity, decreased while its structure was not significantly altered; this is consistent with imposition of geometrical constraints due to multivalent binding, with the toxin A-subunit located away from the lipid interface [78]. Cholera toxin causes a change of the thermotropic behavior of the lipid phase [217–219] and the ganglioside binding has a large enhancing effect on the cooperative interactions within the toxin molecule. While the recognition of the ganglioside GM1 by the toxin is unquestionable, the simplified model used to represent the cholera toxin interaction with the bilayer membrane only through simple specific binding to GM1 exclusively mediating subsequent toxin penetration [220] is not valid. Apart from toxin-induced phase changes in GM1 and GM1-induced toxin stabilization, the binding of cholera toxin to phospholipid bilayer vesicles containing the ganglioside GM1 causes a blue shift of the intact toxin or its B subunit tryptophan emission spectra indicating the location of that residue in a more hydrophobic environment upon binding [217,218]. However, this is not specific for ganglioside GM1 and a similar effect is found with disialoganglioside GD1b together with a same capacity of both gangliosides to reduce the toxin fluorescence quenching by iodide [44]. Direct measurement of the toxin penetration into ganglioside-containing lipid interfaces with a well controlled and known surface organization has shown that its insertion into the interface is not dependent on the ganglioside binding capacity in any simple manner and, furthermore, the presence of ganglioside GM1 is not necessary for the toxin penetration into lipid interfaces [221] Similar conclusions were reached regarding the binding and penetration of tetanus toxin into polysialoganglioside monolayers [222].

Regarding other proteins, it was found that the intermolecular packing and the presence or absence of surface immiscibility in mixed interfaces of various GSLs with MBP, melittin, bovine serum albumin and glycophorin depend largely on the relative proportions and type of protein and on the oligosaccharide chain on the GSLs. When the protein proportion is below 2 mol% the surface is usually homogeneous but has a higher thermodynamic stability to form a collapsed bulk phase or become laterally phase-separated [85]. The latter is abruptly established when the contribution to the surface area by the protein reaches about 30–40%, independently on the mole fraction or the nature of the protein (for relatively large proteins the surface composition corresponds only to a few mol% of protein). The mean molecular area of the GSLs is reduced to its minimum possible value (near the limiting figure of about 0.35–0.40 nm^2 for a closely packed lipid with two hydrocarbon chains) due to the interactions with the protein, this is independent on the size and type of oligosaccharide chain in the GSLs [44,85,211]. Basic proteins penetrate more readily and to a larger extent into interfaces of negatively charged GSLs [211] with changes of molecular packing and dipole potential caused by a complex interplay of different factors. The variations of interfacial micropolarity induced in the GSLs-containing interfaces by the proteins suggested dehydration of the oligosaccharide chain of the more

complex GSLs, apart from the establishment of electrostatic interactions [88].

MBP affects the T_m of synthetic SM [223] while that of DPPC, GalCer and Gg4Cer is little affected and the GSLs calorimetric transition enthalpy decreases in proportion to the protein content. GalCer is more condensed and has a higher T_m than Sulf; interactions with MBP modulates the lipid phase state with the protein inducing a shift to more liquid-like phase state of each lipid [44,224]. The amount of protein needed to induce the maximum shift is about 10 times less for Sulf than for GalCer, reflecting the preferential penetration and interaction of MBP with the former lipid [211]. The phase behavior is further modified by the presence of phospholipid in ternary systems; in mixtures with GalCer and DPPC the protein does not induce segregated domains but partitions preferentially into the liquid–crystallline phase while in ternary systems with Sulf defined phase separation with coexistence of high melting Sulf-rich and low melting Sulf-depleted domains are found[44]; similar behavior was reported for mixtures of DPPC with ganglioside GM1 in the presence of MBP [224]. The perturbed lipids no longer participate in a cooperative phase transition while the remaining GSLs undergo the phase change with the same degree of intermolecular cooperativity indicating the presence of protein-enriched segregated domains with a behavior characteristic of proteins deeply embedded into the lipid bilayer [87,225]. By contrast, the increase of the transition enthalpy and slight increase of T_m induced by MBP on the thermotropic behavior of gangliosides corresponds to a surface mediated protein adsorption through electrostatic interactions [87,225].

Membrane proteins may stabilize and retain acidic GSLs (such as ganglioside GM1) and exclude other GSLs (such as cerebrosides) in compositionally-segregated surface domains, as recently found in monolayers formed with all the lipid and protein components of whole myelin membrane [98]. The segregated domains containing myelin proteins and GM1 have increased optical thickness and show a transition from round-border domains to fractal domains occurring during compression [100]. Liquid–liquid phase immiscibility was known from early calorimetric studies of binary phospholipid systems [226]. For mixtures of different neutral GSLs or gangliosides with DPPC both liquid–liquid and gel–gel phase immiscibility was first revealed by the temperature-composition phase diagrams [139] and was extended more recently to mixtures of short-chain Cers [61,192]. In more complex systems, liquid–liquid surface immiscibility was initially described in monolayers containing CHOL prepared with a total lipid fraction from red blood cell membranes [127]. In monolayers formed with whole myelin we described that at least two types of liquid phase domains coexist (liquid-expanded and liquid-ordered, having relaxed boundary line tension and Brownian motion) at low and high lateral surface pressures [97]. Fluid-phase coexistence was subsequently found in bilayer vesicles made from natural kidney brush border membranes [128] and was recently reported in bilayer vesicles prepared with the natural mixture of lipids and pulmonary surfactant protein [227].

The implication of surface carbohydrates in membrane adhesion was proposed over 35 years ago [228] and evidence was subsequently provided showing the participation of neutral GSLs and gangliosides in cell–cell adhesion [14,229]. The membrane apposition induced by calcium ions, lectins or proteins between bilayer vesicles containing GSLs depend on the length of their oligosaccharide chain [199,200,230]. On the other hand, the interaction of a lectin with GSLs is not only determined by the stereochemical orientation of the terminal sugar residues and the size or conformation of the carbohydrate binding site but also by the properties of the lipid phase which markedly modulate lectin–surface interactions [230,231]. It has been well established that the interaction, adhesion, merging and/or recombination of bilayer membranes consists of the thermodynamically favorable outcome of several and complex balances of forces, among others those derived from surface free energy, membrane curvature, surface electrostatics, hydration, and Van der Waals forces [232]. The oligosaccharide chain of GSLs appears to participate at several levels in the modulation of these tensions [13,43,44,230,233,234].

1.4.3. Biocatalytic-structural cross-talk at the membrane interface

Membrane-associated enzymes, either being those proteins integral to the membrane or the large class of amphitropic proteins that can reversibly interact with membrane surfaces, express activity depending on subtle changes of molecular organization in the protein itself or in the membrane surface [235–237]. This includes practically all types of enzymes that are of paramount importance for membrane signal transduction at the membrane level [235] and whose activity responds to, and in essence regulates, membrane composition and structure at the local biocatalytic level. Although not yet well incorporated in the conceptual framework of biochemistry and cell biology, the two-dimensional surface has an extraordinary capacity to translate with amplification very minor local variations of chemical structure due to the non-linear dissipation of thermodynamic and geometric tensions, resulting in major supramolecular and topological events in turn affecting and regulating membrane metabolism itself. Not surprisingly, the varied effects of GSLs on the membrane structural dynamics have profound influences on the activity of enzymes acting in membranes. This is a key aspect of molecular information exchange at which the varied polymorphism of most membrane lipids, and of GSLs in particular, constitutes a unique membrane phenomena right at the mesoscopic level [13,44]. It represents a multidimensional linking point between the local molecular events of metabolism (which are opened to the interrelated pathways of biochemistry) and the supramolecular membrane dynamics affecting structural recombinations influencing inner and outer cellular communication (with translation to most functions in cell biology). Many examples exist demonstrating a rich participation of GSLs in the control of enzyme activity at the membrane level. Almost 30 years ago it was first demonstrated that gangliosides activated Mg^{2+}-ATPase strongly and Ca^{2+}ATPase slightly in deoxycholate-treated rat brain microsomal fractions [238] while the Na^{+}/K^{+}-ATPase in synaptosomal membranes was inhibited by mixed gangliosides and the Mg^{2+}-ATPase was not found affected [239]. Gangliosides have been shown to affect various protein kinases (calmodulin-

dependent, phospholipid-dependent and others) in different neural systems and cells and both stimulatory and inhibitory effects on protein phosphorylation by different mechanisms were reported [240,241]; for more references see [15].

With respect to neuraminidase, the rates of hydrolysis of gangliosides by the *Vibrio cholerae* enzyme are larger for membrane-bound than for micellar gangliosides [242,243]. Since the sialidase has no recognition site for the ganglioside aglycon [244], the changes of activity must be obviously due to accessibility of the sialosyl residue toward the aqueous medium; therefore, the lateral and/or transverse organization at the interface, with its consequence on oligosaccharide chain orientation come into play. The rate of activity of neuraminidase can be finely regulated by changes of the membrane phase state and the lateral surface pressure, with the enzyme preferring substrates that are more loosely packed. Correlation of the thermotropic behavior of ganglioside-containing phospholipid bilayers with sialidase activity indicated that the rate of ganglioside degradation by the enzyme can be reversibly regulated by the membrane physical state [67,214,215,245]. The rate of hydrolysis is higher when the gangliosides are homogeneously dispersed along the bilayer surface than when they are present in enriched domains while the affinity for the substrate does not appear to be affected; this is in agreement with the preference of the enzyme to degrade gangliosides at lower lateral surface pressure where the lipids are more loosely packed [245]. It is not the formation of the enzyme–ganglioside complex or the initial sialosyl residue recognition that are regulated by the surface organization but subsequent kinetic steps of the reaction such as the formation or liberation of the product in the membrane [215]. A definite lag-time occurs before the onset of a constant rate of hydrolysis indicating precatalytic events taking place before effective enzyme activation, while penetration of neuraminidase into the ganglioside-containing interface can occur without catalythic activity, meaning that protein domains other than the active site are implicated in its interfacial association [245]. In addition, superimposed to the supramolecular effects, local chemical changes such as intramolecular hydrogen bonding between the protonated carboxyl group of sialic acid and the GalNac carbonyl reduce dissociation of the acid and impairs the enzymatic hydrolysis by neuraminidase [246] thus amplifying the effects to the biocatalytic level.

Regarding phosphohydrolytic enzymes, it has long been known that the membrane intermolecular organization and lipid (substrate and non-substrate) composition have a profound influence on the enzyme ability to become membrane-associated, as well as to undergo pre-catalytic or catalytic steps required for the in-plane substrate degradation and several details of their surface regulation were unraveled [56,99,208, 247–253]. Also, it was demonstrated that different phospholipases that do not share common lipid intermediates can cross-communicate on biophysical terms at the interface depending on the type and proportion of product/substrate mutually generated and how it affects the surface topography [99,116,254]. Several GSLs, which are non-substrates for phospholipases, can markedly regulate their activity through changes of the lateral organization, phase state or topology of the membrane; with respect to the latter, it was shown that the rate of activity of PLA_2 and PLC is extremely sensitive to the generation of HII-type of structures in the bilayer membrane and varies according to their formation [37,38,44,255].

Kinetically, there are at least three inter-dependent levels at which the effect of GSLs (and other lipids) exert their modulatory effect on the surface reaction: on the initial adsorption/partition or relocation of the enzyme in the interface; on the enzyme precatalytic activation that frequently determines the length of the latency period for activity; the expression of catalytic activity itself through the rate and extent of product formation. On the other hand, the complexity of the regulatory process represents a multiple transducer device in itself since each of these steps can become rate-limiting in a structure- as well as time-dependent manner that is self-controlled to steady-state, amplification or dampening modes depending on the topological changes at the surface evolving during the enzymatic reaction [37,38,255–258]. Several GSLs can markedly affect phospholipase activity through their effects on the membrane organization at the various levels [13,44]. Neither the association of the PLA_2, PLC and SMase to the interface, nor its affinity for the phospholipid substrate, are impaired by the GSLs or the presence of the reaction products. Rather those lipids generally facilitate interfacial enzyme partition and shorten or abolish the lag-time for precatalytic activation, irrespective on whether their effect is to activate or inhibit the steady-state catalytic reaction [44,56].

The electrostatic field across the interface, either externally applied or locally exerted by the orientation of resultant dipole moment vector due to the different polarity of the membrane molecules, can markedly affect the phospholipase reaction, with the enzyme activity reversibly increasing or decreasing depending on the application of negative or positive, respectively, electrostatic fields on the hydrocarbon chain side of the interface [179,259]. For phospholipids, the positive end of the electrostatic field vector of the resultant molecular dipole moment perpendicular to the interface points toward the hydrocarbon chai*N*-methyl end which means that, at the molecular level, activating fields induce phospholipid hyperpolarization while inhibitory fields imply interfacial depolarization; Several natural and chemically modified derivatives of sphingosine, GSLs or proteins can depolarize or hyperpolarize the interface, with the phospholipase activity responding in concert according to the variations of the interfacial polarization vector and the combined effects of local and externally applied electrostatic fields [44,57,58,179, 237,260]. We recently showed that sphingo- and GSLs adsorbed to carbon electrodes affect in a voltage-dependent manner charge transfer reactions [193] and reversible surface reorganization of GalCer occur with marked hysteresis depending on the sign and magnitude of the electrostatic potential applied [261].

For SMase, ganglioside GM1 moderately inhibits the activity while asialo-GM1 has no significant effect [252]. Catalytic formation of Cer-enriched domains by neutral SMase drives dynamic structural reorganization that controls activity in a bidirectional manner [99]. Real time epifluorescence microscopy of monolayers under the action of SMase provided direct evidence that the enzyme activity continuously alters the surface topography. During the first phase of the enzymatic reaction and up to the end of the latency period phase separated Cer-enriched

domains (and lateral defects) increases rapidly in number until reaching a plateau; this first structural threshold point signals a transduction from the local biocatalytic event to a topographically-mediated switch-on of enzymatic activity to a constant rate regime. During the following pseudo-zero-order catalysis the number of Cer-enriched domains remains unaltered while steadily growing in size. The spatial distribution of domains shows remarkable long-range order and defined lattice superstructuring. In turn, the topographical changes feed back on the local molecular level of the SMase kinetics and a second structural threshold triggers a slowing-down of the catalytic rate and subsequent switch-off of activity. This occurs when the liquid-expanded SM-enriched phase (so far constituting the continuous surface phase) becomes "disconnected" by the percolation of the superstructure of condensed Cer-enriched domains, with increase of surface viscosity [99]. These changes are strongly correlated to the induction of domain shape variations and lattice superstructuring, driven by the electrostatic dipole moment density difference between the coexisting phase domains, balanced by the lateral line tension at the domain boundary; in addition, the inter-domain electrostatic energy in relation to the thermal energy determines domain lattice formation. Additional information content and transduction codes are contained in these phenomena since the surface topography of the mixed interface is dependent on the manner in which the compositional changes are generated: the domain pattern, distribution, and percolation point of Cer-enriched domains are different in enzyme-free films in which SM and Cer are premixed at the same relative proportions than those derived from the catalytic process [99]. In a fluorescence microscopy study with giant bilayer vesicles, SMase was shown to induce budding of vesicles in a vectorial manner depending on the bilayer half in which Cer is being generated [262]. These findings clearly show the extraordinary richness of structural information transduction to various hierarchical levels, extending far beyond purely biochemical cascades, related to the existence of segregated sphingolipid compositional domains.

As a closure, and perhaps as a reminder to avoid dogmatisms, quotation of an enlightening statement may also be pertinent to biophysical research on glycosphingolipid "We, our self-assumed logical rationality that by consensual agreement we decreed as operating within ourselves, have imagined the universe. We dreamed and invented it resistant, visible, mechanical, finite, sequential and causal in time and space, with a glorious defined origin and a hopeful objective which, though admittedly unknown, have designed and assumed to be inherently logical and deterministic. However, we have been wise enough at least to let pervade in the architecture and development of that masterly dream tiny, subtle, eternal and ubiquitous cracks and flaws of folly, mystery, ambiguity, and incomprehension so as to let us perceive that our claimed impressive construction might be profoundly, desperately and unmercifully fictitious" (Jorge Luis Borges[1]).

[1] "Una vindicacion de los gnosticos". Interview and Comments on "Discusión: La perpetua carrera de Aquiles y la Tortuga". Diario La Prensa, Buenos Aires, 4th Seccion. January 1st 1932.

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